The cellular and molecular mechanisms for \( \beta \)-cell loss and dysfunction in type 1 diabetes (T1D) remain unclear. Insulitis has served as a morphological hallmark of T1D but, in contrast to findings in the prevailing animal models of the disease, insulitis in humans seems discrete and composed of only few immune cells predominantly located in the interface between the islets and the surrounding exocrine parenchyma. In subjects with recent-onset T1D, insulitis affects only few islets and is heterogeneously distributed within the gland. In a recent meta-analysis, discrete insulitis was reported in only 56% of subjects examined within the first month after diagnosis of T1D. Phenotypically, CD8\(^+\) T cells dominate the lesion, followed by CD68\(^+\) monocytes/macrophages. CD4\(^+\) T cells and B cells are less frequent, and regulatory T cells, natural killer cells, and plasma cells were found only rarely. No further subtyping of the T cells present in the insulitic lesions of subjects with T1D has been reported.

Despite intense research, the impact of T cells to disease development is under debate, and islet autoreactive CD8\(^+\) T cells in peripheral blood were recently found to be equally frequent in patients with recent-onset T1D when compared to healthy volunteers. Similarly, intervention therapies targeting these cells in subjects with recent-onset T1D have been evaluated.

Subtypes of CD8\(^+\) T cells in insulitic lesions in biopsy specimens from six subjects with recent-onset type 1 diabetes (T1D) and six nondiabetic matched controls were analyzed using simultaneous multicolor immunofluorescence. Also, insulitic islets based on accumulation of CD3\(^+\) T cells were microdissected with laser-capture microscopy, and gene transcripts associated with inflammation and autoimmunity were analyzed. We found a substantial proportion, 43%, of the CD8\(^+\) T cells in the insulitic lesions to display a tissue resident memory T cell (TRM) (CD8\(^+\)CD69\(^+\)CD103\(^+\)) phenotype in T1D subjects. Most TRM cells were located in the insulitic lesion in the endocrine-exocrine interface. TRM cells were also sporadically found in islets of control subjects. Moreover, gene expression analysis showed a lack of active transcription of genes associated with acute inflammatory or cytotoxic T-cell responses. We present evidence that a substantial proportion of T cells in insulitic lesions of recent-onset T1D patients are TRM cells and not classic cytotoxic CD8\(^+\) T cells. Our findings highlight the need for further analysis of the T cells involved in insulitis to elucidate their role in the etiology of T1D.

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cause no, or only transient, preservation of β-cell function. Collectively, these observations emphasize the need for further characterization of T cells in the insulitic lesions in subjects with recent-onset T1D.

Tissue resident memory T cells (T_{RM} cells) constitute a subset of memory T cells defined by their inability to recirculate to lymph nodes and blood as other memory subsets.\textsuperscript{13,14} Hence, these cells persist for years at the site of previous infection and can provide rapid immune protection against reinfection via the same entry port.\textsuperscript{15} Accumulation of T_{RM} cells in peripheral tissues (eg, lesions in psoriasis and vitiligo) can be identified by expression of integrin αE, CD103, and glycoprotein CD69.\textsuperscript{16} In psoriatic lesions, approximately half of epidermal CD8\textsuperscript{+} T cells express these T_{RM} phenotypic markers.\textsuperscript{17,18} Recently, we demonstrated that T_{RM} cells are present in low numbers in pancreatic samples from nondiabetic individuals without inflammation.\textsuperscript{19}

Our previous finding of a distinctly different T- and B-cell gene expression pattern in infiltrated islets of patients with recent-onset T1D when compared with that observed in kidney transplant biopsy specimens with ongoing T-cell–mediated allograft rejection\textsuperscript{2} raised the hypothesis that the T cells found in the insulitic lesions constitute unconventional T cells. A further characterization of the phenotypes of the T cells present in the insulitic lesions from these patients was therefore performed using multicolor immunofluorescence and laser-capture microdissection with subsequent gene expression analysis.

### Materials and Methods

#### Patient Samples

Pancreatic samples from six type 1 diabetic patients, the Diabetes Virus Detection (DiViD) study,\textsuperscript{20} were used in the present study. The DiViD study was approved by The Norwegian Governments Regional Ethics Committee. Written informed consent was obtained from all cases after oral and written information from the diabetologist and the surgeon separately.\textsuperscript{20} Three women and three men, 3 to 9 weeks after diagnosis, at an age between 24 and 35 years, were participating in the study. All patients were insulin dependent, were positive for glutamic acid decarboxylase autoantibodies, and had at least one high-risk human leukocyte antigen haplotype. Detailed clinical characteristics are described by Krogvold et al.\textsuperscript{20} Pancreatic tail biopsy specimens from six nondiabetic deceased organ donors, matched for age (range, 20 to 37 years; mean, 25.8 years), body mass index (range, 20.1 to 29.4 kg/m\textsuperscript{2}; mean, 25 kg/m\textsuperscript{2}), and sex, served as controls. Consent for organ donation (for clinical transplantation and for use in research) was via online database (https://www.socialstyrelsen.se/donationsregistret/anmalan) or obtained verbally from the deceased’s next of kin by the attending physician and documented in the medical records of the deceased in accordance with Swedish law and as approved by the Regional Ethics Committee (Dnr 2015/444).

#### Immunofluorescence

Biopsy specimens frozen in liquid nitrogen and kept at −80°C were cut into sections (10 μm thick) and fixed in 1% methanol-free paraformaldehyde (Sigma Aldrich, Darmstadt, Germany). Blocking was performed with 5% normal goat serum in tris-buffered saline supplemented with 0.05% Tween-20 (Dako, Glostrup, Denmark). Thereafter, sections were incubated with primary antibodies; details can be found in Table 1. After rinsing of unbound antibody, the sections were incubated with appropriate secondary antibodies raised in goat (dilution, 1:600) for 1 hour at room temperature, as shown in Table 1. Finally, sections were counterstained with DAPI (300 nmol/L; Life Technologies, Carlsbad, CA) and further processed for image analysis. Fluorescent signals were visualized using a confocal microscope (LSM700; Zeiss, Oberkochen, Germany). Human spleen and tonsil were used as positive controls. Omission of primary antibody served as negative control.

#### Imaging and Data Analysis

The whole insulin and glucagon stained section was scanned for each patient. This enabled identification of insulin-containing islets and insulin-deficient islets. In consecutive

<table>
<thead>
<tr>
<th>Name</th>
<th>Host</th>
<th>Dilution</th>
<th>Clone</th>
<th>Supplier</th>
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<td>Dako</td>
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<tr>
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</tr>
<tr>
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<td>1:400</td>
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<td>Life Technologies</td>
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TCR, T-cell receptor.
sections, these islets were triple stained for CD8, CD69, and CD103, and cells located in the endocrine-exocrine interface and within the islet parenchyma were counted manually using ImageJ software version 2.0.0 (NIH, Bethesda, MD; http://imagej.nih.gov/ij). All islets in each section were analyzed, and the number of CD8 and $T_{RM}$ cells in each islet is presented.

**Laser-Capture Microdissection and Transcriptome Analysis**

RNA isolated from the same microdissected previously from pancreatic tissue was used in this study. Briefly, frozen tissue samples from the pancreatic tail region were cut into sections and mounted onto Superfrost Plus glass (Menzel-Gläser, Braunschweig, Germany) or Arcturus PEN Membrane Glass Slides (Life Technologies) for immunohistochemistry and laser-capture microdissection, respectively. Consecutive sections were stained for CD3 or used for laser-capture microdissection to microdissect islets with insulitis ($\geq 15$ CD3$^+$ cells) from the diabetic samples and islets without insulitis from the nondiabetic samples. cDNA synthesis, preamplification of cDNA, and expression analysis was performed with kits from Qiagen (Sollentuna, Sweden), as described. A pathway-specific primer mix (Human Inflammatory Response and Autoimmunity, PBH077Z; Qiagen) was used for the preamplification, and a PCR array (Inflammatory Response and Autoimmunity, PAHS-077Z; Qiagen) was used for the expression analysis of 84 genes involved in inflammation. The expressions of β-actin ($ACTB$), glyceraldehyde 3-phosphate dehydrogenase ($GAPDH$), and 60S acidic ribosomal protein P0 ($RPLP0$) were used for normalization.

**Statistical Analysis**

Statistical differences between the groups were calculated using Mann-Whitney U test using GraphPad Prism software version 6 (La Jolla, CA), and $P \leq 0.05$ was considered significant. The number of islets analyzed per subject is stated in the respective figure legend.

**Results**

CD8$^+$ T cells were the most abundant T-cell phenotype in the recent-onset T1D subjects. The number of CD8$^+$ T cells per islet was similar in four of six patients, whereas islets in patients 5 and 6 had markedly more CD8$^+$ T cells (Figure 1A). Most of the CD8$^+$ T cells were located in the endocrine-exocrine interface. When all islets from T1D subjects were analyzed and categorized on the basis of their insulin positivity, we found a significantly higher ($P = 0.0059$, Mann-Whitney U test) number of CD8$^+$ T cells per insulin-containing islet ($n = 26$; median, 9 cells) compared to insulin-deficient islet ($n = 20$; median, 3 cells) (Figure 1B).

Simultaneous staining for CD8, CD69, and CD103, markers expressed by $T_{RM}$ cells, revealed a CD8$^+$CD69$^+$CD103$^+$ population, as shown in Figure 2, A–C, demonstrating the presence of a substantial proportion of $T_{RM}$ cells in insulitic lesions of recently diagnosed T1D patients. $T_{RM}$ cells were sporadically found within the islet parenchyma but in markedly fewer numbers when compared with the $T_{RM}$ cells located in the exocrine and endocrine interface (Figure 2, A–C).

Quantification of CD8$^+$CD69$^+$CD103$^+$ cells in the pancreas of T1D patients shows that on average 43% of all CD8$^+$ T cells per islet had the phenotype of $T_{RM}$ cells and not conventional recirculating memory T cells (Figure 2D). Although expected variations between the six DiViD subjects as well as between individual islets were observed, 35 of 37 (94.6%) analyzed islets with one or more CD8$^+$ cells contained one or more CD8$^+$CD69$^+$CD103$^+$ cell.

The proportion of $T_{RM}$ cells of all CD8$^+$ T cells did not differ between insulin-containing islets and insulin-deficient islets (Figure 2E). More important, among the CD8
population analyzed, no CD103− cells were CD69+, suggesting absence of recently activated conventional CD8+ T cells in the insulitic lesions of T1D subjects. γΔ T cells were observed only rarely in the pancreas from T1D subjects, in the endocrine-exocrine interface or in the pancreatic parenchyma.

Although markedly fewer compared to T1D subjects, CD8+CD69+CD103+ cells were also found in islets of control subjects (Figure 3), which corroborates a resident T-cell phenotype. More specific, of 44 islets examined, 21 (48%) had at least one CD8+ T cell (Figure 3); 19 of these 21 CD8 T-cell containing islets had one or more CD8+CD69+CD103+ TRM cells (Figure 3), suggesting minimal patrolling of recirculating T cells.

Expression analysis of genes associated with inflammation and autoimmunity of insulitic islets revealed that 19 genes of the 84 genes in the array were not detected in insulitic islets of any T1D subjects and several were at the border of detection limit (Figure 4A). Surprisingly, the expression of genes associated with an acute cytotoxic response, such as TNF, CCR7, LTA, and IL1A or activated cytotoxic T cells (IL2R, CD40LG, and FASLG), were not detected in any T1D subjects. However, genes encoding cytokines more related to inflammation and innate immune...
responses (e.g., IFNG, IL18, IL22, and IL15), chemokines, and ligands CXCL1, CCL11, CXCL9, CCL19, CCL7, CCL5, and CCL4 were up-regulated in insulitic islets of T1D subjects compared to noninsulitic islets from nondiabetic control donors, as shown in Figure 4, A and B.

Discussion

Our understanding of different T-cell subsets has markedly increased during the previous decade. Herein, we present evidence supporting the presence of a substantial proportion of T_{RM} cells in the insulitic lesions in biopsy specimens from adult subjects with recent-onset T1D. In fact, T_{RM} cells were found in pancreata of all T1D subjects from the DiViD cohort and represent on average approximately 40% of the total number of CD8\(^+\) T cells per islet, a proportion of T_{RM} cells similar to that previously described in skin lesions of psoriasis.\(^{18}\)

These findings, together with the relatively low prevalence of insulinitis,\(^{2,7}\) in recent-onset T1D subjects and the fact that most of the T cells are residing in the islet-exocrine interface,\(^{2,5,6}\) have implications for the prevailing view on the etiology of T1D, encompassing a crucial role for autoreactive cytotoxic CD8\(^+\) T cells specifically targeting the insulin-producing cells.\(^{25,26}\) Presence of autoreactive CD8\(^+\) T cells in islets of T1D subjects was shown by Coppieters et al\(^{25}\); however, numbers of CD8\(^+\) T cells were low, with no difference between subjects with recent-onset T1D and subjects with long-standing T1D. Also, because no quantification, nor further subtyping or assessment of activation, of the autoreactive CD8\(^+\) T cells in respective subjects was performed, the specificity and phenotype of most insulin T cells remain unknown.

In vitro characterization of circulating autoreactive CD8\(^+\) T cells shows ultra-low-affinity binding to the islet autoantigens and huge promiscuity.\(^{26,27}\) Despite these observations, these cells are able to kill human β cells in vitro.\(^{26,28}\) However, the authors conclude that “such weak agonists will not generally be physiologically significant unless presented at very high copy numbers.”\(^{27, p.1175}\) In line with these observations, analysis in peripheral blood revealed autoreactive CD8\(^+\) T cells to be equally frequent in patients with recent-onset T1D and matched healthy volunteers,\(^{8}\) suggesting that the presence of a small number of autoreactive T cells is not sufficient for initiation of an autoimmune attack and development of T1D.

Experimental evidence clearly indicates that T_{RM} cells are left remaining at the site or in close vicinity of previous infections in the absence of ongoing antigen presentation as a means to rapidly mobilize and recall an immune response if reinfection occurs via the same entry port.\(^{29,30}\) The substantial proportion of T_{RM} cells in the islets of recent-onset T1D subjects reported herein may suggest involvement of an infectious agent in the development of T1D\(^3,31–33\) and a

Figure 3  Tissue resident memory T cells (T_{RM} cells) in pancreata of nondiabetic donors. A: Confocal images of islets from four different nondiabetic control subjects stained for CD8 (green, AlexaFluor 488), CD103 (yellow, AlexaFluor 555), and CD69 (red, AlexaFluor 647) and DAPI. Islets are indicated with a dashed line, and arrows indicate T_{RM} (CD8\(^+\)CD69\(^+\)CD103\(^+\)) cells. B and C: The number of CD8\(^+\) T cells (B) and the proportion of CD8\(^+\) T cells with a T_{RM} phenotype (C) are displayed per islet in each of six nondiabetic controls. Each dot represents an individual islet. Data are presented as individual values with medians. Scale bar = 100 μm (A). C1 to C6, controls 1 to 6.
significant role of TRM cells. However, a causative infectious agent in T1D remains to be demonstrated.

The function of human TRM cells is so far best described in psoriasis skin lesions, where CD8⁺CD103⁺ TRM cells, when restimulated, show potent effector function by significant production of interferon-γ, tumor necrosis factor-α, IL-17, IL-22, and IL-4 compared to recirculating T cells, but exhibit a lower proliferative capacity compared to the CD103⁺ resident population. In addition, inflammatory genes remained up-regulated in resolved lesions at least 3 months after systemic anti-tumor necrosis factor-α treatment.

From our data, it is not possible to define the functional status of the TRM cells because microdissection was not...
selectively based on \( \text{T}_{\text{RM}} \) cells. However, in line with previously reported data, \( \text{FASLG} \), \( \text{TNF} \), and \( \text{CD154} \) could not be detected in TID subjects and, together with the lack of CD8\(^+\)CD69\(^+\)CD103\(^+\) T cells, in the insulitic lesions, our findings argue against an ongoing conventional cytotoxic CD8 T-cell response. Although it is not feasible to directly translate the gene expression data to the \( \text{T}_{\text{RM}} \) cells found in the insulitic lesions, it is intriguing to speculate that the levels of IFNG, IL22, IL15, and IL18 observed are because of low-grade activation of the \( \text{T}_{\text{RM}} \) cells present in the islet-exocrine interface. Nevertheless, contribution from other cell types cannot be neglected.

The diabetic pancreata included in this study were from adult subjects diagnosed with type 1 diabetes based on clinical and laboratory (presence of autoantibodies) criteria. It has been shown previously that the insulin profile differs between adults and children diagnosed with T1D. Whether \( \text{T}_{\text{RM}} \) cells are a general feature of the insulitis in T1D, or if it defines only a subtype of T1D patients diagnosed as adults, remains to be determined.

In summary, we present data demonstrating the presence of a substantial proportion of \( \text{T}_{\text{RM}} \) cells, a subtype of T cells not previously described in the insulitic lesions of T1D. Our findings suggest that a large proportion of the CD8\(^+\) T cells present in the insulitic lesions, at least in adult patients, are part of a protective, not cytotoxic, immune response. This study highlights the need for further characterization of the insulitic lesions, to elucidate the role of these cells in the etiology of T1D and to allow development of effective means to intervene in disease progression.

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E.K. participated in the study design, performed experiments, data analysis, and interpretation, and wrote the manuscript; P.S. performed the experiments, data analysis, and interpretation and participated in writing the manuscript; L.K. was responsible for clinical coordination, the recruitment of patients, and data collection, analysis, and interpretation, and participated in writing the manuscript; B.E. and T.B. performed the surgery and participated in writing the manuscript; K.F.H. was involved in the coordination of the DiViD study and participated in writing the manuscript; K.D.-J. is the principal investigator of the DiViD study and, as such, was responsible for design of the DiViD study, funding, regulatory issues, and international collaboration and participated in writing the manuscript; O.S. participated in the study design, data interpretation, and writing of the manuscript; O.K. designed the study, interpreted the data, and wrote the manuscript; O.K. is the guarantor of this work and had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

References


